

Brazilian Journal of Veterinary Research and Animal Science (2004) 41:379-383  
ISSN printed: 1413-9596  
ISSN on-line: 1678-4456

## Anti-human influenza protector antibody detected in horses as a zoonotic viruses

## Anticorpo protetor anti influenza humana detectado em cavalos, como virose zoonótica

Dalva Assunção Portari  
MANCINI<sup>1</sup>;  
Rita Maria Zucatelli;  
MENDONÇA<sup>1</sup>;  
José Ricardo PINTO;  
Enio MORI<sup>2</sup>;  
Wilson Roberto FERNANDES<sup>2</sup>

1- Laboratório de Virologia do Instituto Butantan, São Paulo - SP  
2- Departamento de Clínica Médica da Faculdade de Medicina Veterinária e Zootecnia da USP, São Paulo - SP

### Abstract

This study aimed to investigate the incidence of the influenza virus, and its interspecies transmission cycle among horses. A comparative serological survey was performed using horse sera following challenge with both specific (equine) and non-specific (human) influenza virus strains. Bleedings of the 22 horses were performed during the years 1999 and 2000. Following treatment with kaolin (20%), added in rooster erythrocytes suspension (50%), for removal of non-specific antibodies, sera were titrated against both Human and Equine Influenza virus, by the Hemagglutination Inhibition Assay (HI), recommended by WHO. The HI results of horse serological responses demonstrate cross reaction between both the specific strain, A/Eq1 (H7N7) (62.75%) and A/Eq2 (H3N8) (60.65%), and the non-specific strains, type A (H1N1) (79.5%) and A (H3N2) (94.45%) and type B (77.75%). It was noteworthy the high percentage of protection responses in equine sera against the non-specific strains, as compared with the specific strains. This finding suggests direct interspecies transmission of influenza virus as zoonotic viruses, particularly for the type B strain which is considered restricted to humans. It was the first report, in Brazil. Further studies are required to achieve a complete understanding of the incidence of influenza in our environment.

### Key-words:

Equine influenza.  
Human influenza.  
Hemagglutination  
Inhibition test.  
Cross-reaction.  
Protection response.

### Correspondence to:

DALVA ASSUNÇÃO PORTARI MANCINI  
Laboratório de Virologia  
Instituto Butantan  
Avenida Vital Brazil, 500  
São Paulo - SP  
labvirol@butantan.gov.br

Received: 21/05/2003  
Accepted: 24/05/2004

### Introduction

The most common virus associated with acute Infectious of Upper Respiratory Tract Disease (IURD), in horses, is the influenza virus.<sup>1</sup> This virus was isolated from horses for the first time in 1956, in Prague (Czechoslovakia) and was identified as A/Eq/Prague/1/56 (Heq1Neq1). In 1963, a new strain of equine influenza was reported in Miami (USA) and named A/Eq/Miami/1/63 (Heq2Neq2). Today, these equine strains are denominated H7N7 and H3N8, respectively, by the WHO Expert Committee.<sup>2</sup>

In Brazil, equine influenza was reported in São Paulo State, in 1988, by our group.<sup>3</sup> In 1969, 1976 and 1986, this virus was also isolated from horses in Rio de Janeiro State. The latter report presented the observation of an antigenic variation of the A/Eq2 Brazilian strain

compared with the reference strain, A/Eq/Miami/1/63 (H3N8).<sup>4,5</sup>

The existence of antigenic variations among equine H3N8 influenza virus hemagglutinins in horses from different hemispheres has been recently reported<sup>7,13</sup>. Although the equine influenza virus is believed to be stable, its antigenic variations have been observed in infected horses imported from different countries. Virus stability is probably associated with the specificity of the circulating strains in each country. Alternatively, direct infection of horses by avian influenza strains may occur, without virus genetic reassortment, since the equine influenza virus is derived primarily from birds.<sup>6,7</sup> The appearance of a new totally unknown emergent strain of influenza virus in horses may act as a warning with regarding to what could happen to the human species. The human immune system would certainly not to

be able to recognize the virus and a viral disease would be inevitable to this species.

Infections of the influenza virus occurring in a direct manner from the avian to mammalian species via the natural hosts have been reported. Scientists have been frequently suggested that the pigs are responsible for the virus genetical reassortment that facilitates avian influenza virus adaptation to mammalian species.<sup>10,11</sup>

Although the type A influenza virus is very easily spread in avian and mammalian species, type B is reported only in a few animal species, such as monkeys and, more recently, in seals.<sup>12,13,14</sup> This fact suggests a threat to humans, since reservoirs facilitate viral genetic recombinations, as currently occurs with influenza A, making it the most epidemic virus.

Due to its minor circulation among the animal species, the type B influenza virus is currently considered the more stable influenza virus. The genetic variation of this virus, which remains poorly understood, does not seem contribute greatly to its evolution.<sup>13,15,16</sup> Lai et al.<sup>6</sup>, demonstrated similarities between type B and equine 2 influenza with regarding to their evolutionary pathways, as the result of slower antigenic drift. Thus, more studies concerning the maintenance of influenza in natural hosts are required, particularly to investigate the possibility that this virus has itself become a zoonotic virus. By definition, the zoonosis is a disease provoked by the same agent either in humans or vertebrate animals. Viral zoonosis have been emerging and re-emerging throughout history and cause serious disease outbreaks in human populations. Emerging viral zoonotic infections with hosts in native species are a hazard for humans. In the past and now, the rabies virus has presented such a problem and, currently, the influenza and hanta viruses have been proposed to form part of this infections group<sup>17</sup>.

“New” viral diseases can arise when viruses broaden their host range and can develop high mutation rates, type A influenza is an example of such a virus.<sup>18</sup> A serological survey realized in Lagos and Ibandan, Nigeria, 1990, showed that 87% of horses responded to the human influenza type A(H3N2).<sup>18</sup> In this study, higher levels of antibodies against human

influenza types A and B were detected in horse sera. Such a finding has also been observed in primates such as non-human (monkeys).<sup>12</sup>

The investigation on the incidence of the influenza virus and its interspecies transmission among horses represented the objective of this work.

## Material and Methods

### Animals

Twenty-two adult horses (*Equus caballus*) were utilized in the study. One group that was vaccinated with bivalent equine influenza vaccine, and another that was not vaccinated against influenza, were evaluated by a serological test. The animals were kept in the Clínica Médica da Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo during the time of experiments.

### Serum samples

Samples were collected from the external jugular vein of the horses, using the vacuum system and disposable needles (40 X 9 mm). After blood coagulation and retraction, the samples were centrifuged (Baby Excelsa 206/I centrifuge) at 260g for 15min. The serum samples were inactivated at 56°C for 30min and treated with Kaolin (20%) added to rooster blood (50%) for removal of non-specific antibodies. The samples were kept at -70°C until the time of the serological test.

### Serology – hemagglutination inhibition test (HI)<sup>15</sup>

This test is based on the inhibition of the hemagglutinating action of the virus by specific antibodies. After treatment, duplicate dilutions of sera were carried out in series, in “V” bottom microplates. Antigens of human and equine influenza virus, cultivated in MDCK cells, containing 4 hemagglutinating units, was added to the cavities. After one-hour of reaction at room temperature, 5% rooster erythrocytes were added to them. Reading was processed after 30 min. The reciprocal of the last dilution which elicited the hemagglutination inhibition was considered as the antibody titer. Those sera presenting antibody titers equal or superior to 1/20 were considered

**Table 1**

Influenza IH titers detected in serum of horses vaccinated with equine influenza virus(A/Eq1 and A/Eq2)

Animals	Strains of Influenza virus					
	Eq1	Eq2	H1N1	H3N2	B/90	B/83
1(Mirinda)	290.9	360	392.7	309.1	226.8	70.9
2(Zarolho)	208	192	44	48	618.7	76
3(Lunga)	344.2	592.7	0	72.7	296	47.3
4(Zito)	160	160	160	80	533.3	106.7
5(Joana D'Ar)	40	40	266.7	40	160	40
6(Lamban)	0	10	320	80	40	0
7(Dida)	40	13.3	80	40	93.3	6.7
8(Bambam)	53.3	93.3	66.7	66.7	333.3	66.7
9(Alamis)	46.7	26.7	66.7	73.3	173.3	40
10(Dodge)	53.3	93.3	80	80	280	40
11(Conan)	93.3	93.3	66.7	40	693.3	66.7
12(Grizza)	80	160	80	320	40	160
13(Castanho)	53.3	77.1	140	93.3	110	60
medium/titers	112.53	147.05	135.65	103.31	276.76	60.07

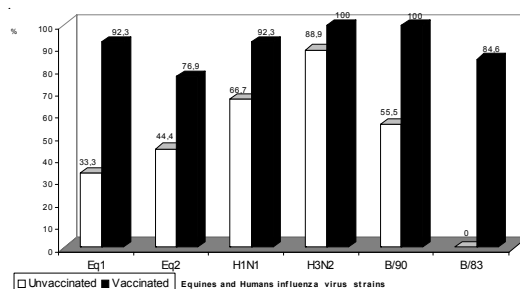
Eq1:A/Eq1/SP/56(H7N7); Eq2:A/Eq2/SP/85(H3N8); A/SP/1/91(H1N1) A/SP/2/95(H3N2); B/90 B/SP/1/90; B/83: B/URSS/83(from Bethesda,MA,USA)

**Table 2**

Influenza IH titers detected in serum of unvaccinated horses with equine influenza virus

Animals	Strains of Influenza virus					
	Eq1	Eq2	H1N1	H3N2	B/90	B/83
14(Malaga)	0	0	142.2	82.2	0	0
15(Tupa)	90.8	346.7	32.7	660	170	25
16(Treca)	0	0	680	120	0	0
17(Bady)	0	106.7	0	56.7	23.3	0
18(Principe)	0	80	290	280	160	0
19(Garimpo)	0	13.3	53.3	146.7	46.7	0
20(Alazio)	46.7	55	80	80	200	20
21(Vivi)	40	0	20	20	160	0
22(Tordilho)	0	0	113.3	70	0	0
medium/titers	19.72	66.85	156.83	168.4	84.44	5

Eq1:A/Eq1/SP/56(H7N7); Eq2:A/Eq2/SP/85(H3N8); A/SP/1/91(H1N1) A/SP/2/95(H3N2); B/90: B/SP/1/90; B/83: B/URSS/83(from Bethesda,MA,USA)

**Figure 1**

Percentage of equines with protector antibody titers against influenza virus

as positive and titers<sup>5</sup> 40HIU were considered protector antibody. For all components the volume of 0.025ml was constant.

### Virus

Influenza virus strains, isolated from human and horses in the city of São Paulo, State of São Paulo, Brazil, were maintained in MDCK cell cultures through successive passages. The following human and equine influenza strains were used: A/SP/1/91(H1N1), A/SP/2/95(H3N2), A/Eq1/SP/56(H7N7), A/Eq2/SP/1/85(H3N8), B/SP/1/90. The B/URSS/83 (from Bethesda, MA, USA), was the standard strain used.

### Results

Table 1 and table 2 demonstrate the results obtained by the HI test to show the responses of the serum horse to the specific strains (Table 1) of influenza virus, as well as the non-specific strains (Table 2). Data shown in Table 1 refer to the animals vaccinated against either A/Eq1(H7N7) or A/Eq2(H3N8) equine influenza strains. HI antibody levels against specific strain components of the equine influenza vaccine are represented by the mean titer detected in horse sera; 112.53HIU for A/Eq1/SP/1/1956(H7N7) and 147.05HIU for A/Eq2/SP/1/1985(H3N8). Strains of human influenza virus antibody titers were 135.65HIU for A/SP/1/1991(H1N1), 103.31HIU for A/SP/2/1995(H3N2), 60.07HIU for B/URSS/1983 (Bethesda, MA, USA) and 276.76HIU for B/SP/1/1990. Table 2 shows the mean titers detected in the sera of horses which did not receive the vaccine against any strains of equine and human influenza virus; 19.72HIU for A/Eq1/SP/1/1956 (H7N7), 66.85HIU for A/Eq2/SP/1/1985 (H3N8), 156.83HIU for A/SP/1/1991 (H1N1), 168.4HIU for A/SP/2/1995 (H3N2), 5HIU for B/URSS/1983 (Bethesda, MA, USA) and 84.44HIU for B/SP/1/1990.

The percentages of horses presenting HI protector antibodies (<sup>5</sup>40HIU), for both specific and non-specific strains of influenza virus are demonstrated in the figure 1.

## Discussion and Conclusion

The results obtained from the serological evaluations realized in this study demonstrated that horses respond to the specific influenza strains (equine) as well as to non-specific influenza (humans) strains. When the group of vaccinated animals (Table 1) was evaluated, the mean titer of antibodies to the influenza virus were almost equivalent for both strains. One horse, however, did not respond to the equine strains, but did to the human strains of influenza virus.

The unvaccinated horses (Table 2) presented lower antibody titers in response to the equine strains compared with the vaccinated group. However, these animals had higher mean antibody titers against the human influenza strains than against the specific equine strains. These data suggest that the horses have been more exposed to human influenza, probably by their feeders. In this group, a prevalence of the A(H3) subtype, originated from humans as well as from horses, was observed. Just two animals did not respond to the human influenza strains, one did not respond to the A(H1N1) strain and one did not have a protector antibodies level to any human influenza type A strain. The latter animal, however, did have a response to the type B strain (titer  $3160$  HIU) and with A(H7N7) (titer  $340$  HIU).

It is important to highlight the low prevalence of the equine strain A/Eq1(H7N7), which showed a mean titer of just  $19.72$  HIU or mean protector antibody level of  $33.30\%$  in unvaccinated horse sera (Table 2, Figure 1). These results are in agreement with a recent report demonstrating a low circulation of the A(H7N7) strain in equine populations during serological survey.<sup>19</sup>

Taking into account the high percentages of protector antibodies against influenza viruses (titer  $340$  HIU), including the type B virus, detected in these animal sera (Figure 1), it is possible to deduce which of these horses were

constantly stimulated by these viruses, independently of vaccination. Similar findings by Olaleye et al.<sup>18</sup>, who reported the presence of human influenza virus in horses during a serological survey realized in Nigeria, in 1999.

However, little current data regarding this subject can be found in the literature. In addition to the studies cited, various authors postulate a theory of direct interspecies infection with influenza virus, between horses and humans.<sup>10,18</sup> Authors further suggest that the influenza virus acts as a zoonotic agent to facilitate its propagation and perpetuation in nature. Nowadays, the type B virus is also cited as participating in the animal group which acts as reservoir for influenza, this group includes monkeys and seals.<sup>3,12</sup>

In addition to the cross-reaction (Ab till  $40$  HIU) with both equine and human influenza type A, the horses evaluated serologically in this study, also presented protection (Ab= $40$  HIU) against both assayed non-specific or specific strains. These animal sera were also able to recognize the influenza virus type B/90, which circulates in Brazil, that it was confirmed by the standard B/83, (from Bethesda, MA, USA) (Figure 1). Once more, these results revealed that the human strains were introduced into the horses through direct interspecies transmission. This fact leads to suggest the zoonotic influenza occurrence. Thus, studies of the ecological properties of influenza may be useful for to further understanding of this viral disease and should aid on its prevention into the human species in the future.<sup>10</sup> In order to evaluate the distribution of the influenza virus in our environment, a search for this virus in other natural hosts is being carried out.

## Acknowledgements

The authors are grateful to the firm VENCOFARMA and Mrs Luzia da Purificação.

## Resumo

O objetivo deste estudo foi investigar em cavalos, a incidência do vírus influenza e seu ciclo de transmissão interespecies. Portanto, levantamento sorológico foi realizado em

### Palavras-chave:

Influenza equina.  
Influenza humana.  
Teste da Inibição da Hemaglutinação.

soro de cavalos, confrontados com ambas cepas, as específicas (equino) e não específicas (humana) deste vírus. Sangrias de cavalos realizadas nos anos de 1999 e de 2000, forneceram soros que, após tratamento com Caolim (20%) e hemácias de galo (50%) para remoção dos anticorpos inespecíficos, foram titulados contra ambas referidas cepas, através do teste de Inibição da Hemaglutinação (recomendado pela OMS). Os resultados, demonstraram que as respostas sorológicas dos cavalos apresentaram reação cruzada entre as cepas específicas e as não específicas. As porcentagens de títulos IH obtidos foram de 62,75% e de 60,65% para as cepas específicas A/Eq1 (H7N7) e A/Eq2 (H3N8), respectivamente. E às cepas não específicas essas porcentagens foram de: 79,05% para A (H1N1), de 94,45% para A (H3N2) e de 77,75% ao tipo B. O mais relevante nestes dados comparativos com vírus influenza, foi a alta porcentagem de resposta protetora à cepa não específica comparada àquela específica, detectada nos soros equinos. Considerando o fato de que o tipo B, deste vírus, ser restrito à espécie humana, portanto a resposta de proteção nos cavalos sugere uma direta transmissão interspécies, como em viroses zoonóticas. Os autores relatam pela primeira vez este tipo de evento no Brasil.

Reação Cruzada.  
Resposta de Proteção.

## References

- MUMFORD, E. L. et al. Monitoring and detection of acute viral respiratory tract disease in horses. **Journal of the American Veterinary Medical Association**, v. 213, n. 3, p. 385-390, 1998.
- WORLD HEALTH ORGANIZATION. Expert Committee. A revised system of nomenclature for influenza A viruses. **Bulletin of World Health Organization**, v. 45, n. 119, p. 123, 1971.
- MANCINI, D. A. P.; GERALDES, E. A.; PINTO, J. R.; SOARES, M. A. An outbreak of influenza in equines of São Joaquim farm, São Roque, SP, Instituto Butantan. **Revista da Faculdade de Medicina Veterinária e Zootecnia São Paulo**, v. 25, n. 1, p. 93-100, 1988.
- CUNHA, R. G.; PASSOS, W. S.; VALLE, M. C. C. Surto de gripe equina produzido por vírus influenza A/Eq1 no Estado do Rio de Janeiro, Brasil. **Revista Brasileira de Biologia**, v. 38, p. 549-554, 1978.
- CUNHA, R. G. et al. Surto de gripe equina produzida por vírus influenza A/Eq2, no Estado do Rio de Janeiro, Brasil. **Revista Brasileira de Medicina Veterinária**, v. 8, p. 88-91, 1986.
- LAI, A. C. et al. Diverged evolution of recent equine-2 influenza(H3N8) viruses in the Western Hemisphere. **Archives of Virology**, v. 42, n. 6, p. 1063-1074, 2001.
- OZAKI, H.; SHIMIZU-NEI, A.; SUGITA, S.; SUGIURA, T.; IMAGAWA, H.; KIDA, H. Antigenic variation among equine H3N8 influenza virus hemagglutinins. **Japanese Journal of Veterinary Research**, v. 48, n. 4, p. 177-186, 2001.
- GREISER-WILKE, I.; HAAS, L. Emergence of "new" viral zoonoses. **Deutsche Tierärztliche Wochenschrift**, v. 106, n. 8, p. 332-338, 1999.
- ILOBI, C. P. et al. Direct sequencing of the H A gene of clinical equine H3N8 influenza virus and comparison with laboratory derived viruses. **Archives of Virology**, v. 143, n. 5, p. 891-901, 1998.
- WEBSTER, R. G. et al. Evolution and Ecology of influenza A viruses. **Microbiological Reviews**, v. 56, n. 1, p. 152-179, 1992.
- WEBSTER, R. G. The importance of animal influenza for human disease. **Vaccine**, v. 20, p. 516-520, 2002. Supplement 2.
- HAGURI, L. C. A. G. et al. Virus respiratórios de origem humano em uma colônia de Mono rhesus(Macaca mulatta). Vírus Influenza –I. **Animales de Experimentacion, La Revista Hispanoamericana**, v. 5, n. 1, p. 11-13, 2000.
- MAC CULLERS, J. A.; WAG, G. C.; HE, S.; WEBSTER, R. G. Reassortment and insertion-deletion are strategies for the evolution of influenza B viruses in nature. **Journal of Virology**, v. 73, n. 9, p. 7343-7348, 1999.
- OSTERHAUS, A. D. et al. Influenza B virus in seals. **Science**, v. 288, n. 5468, p. 1051-1053, 2000.
- PETERHANS, E.; ZANONI, R.; BERTONI, G. How to succeed as a virus: Strategies for dealing with the immune system. **Veterinary Immunological and Immunopathology**, v. 72, n. 1-2, p. 111-117, 1999.
- SUAREZ, D. L. Evolution of influenza viruses. **Veterinary Microbiology**, v. 74, n. 1-2, p. 15-27, 2000.
- van-der-POEL, W. H. "Emerging" viral zoonoses in native animals who can be the host in Netherlands. **Tijdschrift voor Diergeneeskunde**, v. 124, n. 1, p. 3-11, 1999.
- OLALEYE, O. O. et al. Hemagglutination inhibition(HI) antibodies against strains of influenza A virus in horse and pig sera in Nigeria. **Journal of Hygiene Epidemiology Microbiology and Immunology**, v. 34, n. 4, p. 365-370, 1999.
- GUO, Y. et al. Characterization of a new avian-like influenza A virus from in China. **Virology**, v. 188, n.1, p. 245-255, 1992.
- SCHILD, G. C.; PEREIRA, M. S.; CHAKRAVERTY, P. Single- radial haemolysis: a new method for the assay of antibody to influenza haemagglutinin. **Bulletin of World Health Organization**, v. 52, p. 43-50, 1975.